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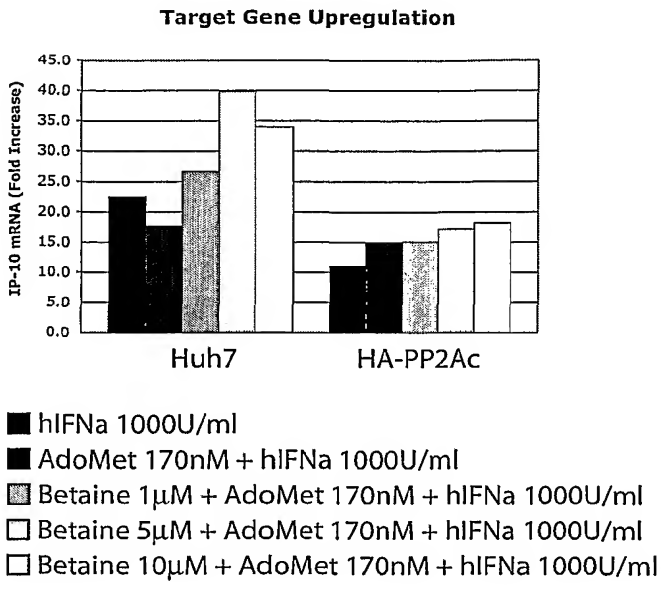

(57) Abstract: The present invention pertains to the signaling involved in suppression of immune function during hepatitis C infection. More specifically, this invention relates to the discovery that PP2A is involved in the inhibition of interferon alpha signaling in hepatitis C infection. Reduced STAT1 methylation and reduced interferon signaling. Restoring methylation by treatment with a methyl group donor increases interferon signaling.

WO 2005/071101 A1

TREATMENT OF HEPATITIS C INFECTION BY INCREASING STAT1 METHYLATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit under 35 U.S.C. 119(e) of U.S. Provisional Application No. 60/560,728, filed January 23, 2004, the entire disclosure of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention pertains to the signaling involved in suppression of immune function during hepatitis C infection. More specifically, this invention relates to the discovery that PP2A is involved in the inhibition of interferon alpha signaling in hepatitis C infection. Reduced STAT1 methylation and reduced interferon signaling. Restoring methylation by treatment with a methyl group donor increases interferon signaling.

2. Background Art

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. An important and striking feature of hepatitis C is its tendency towards chronicity. In over 70% of infected individuals, HCV establishes a persistent infection over decades that may lead to cirrhosis and hepatocellular carcinoma. In order to do so, HCV must escape the host immune response. Type I interferons (IFN) are crucial and potent components of the early host response against virus infection[1]. Many viruses have evolved strategies to protect themselves against the IFN system[2]. Viruses can interfere with the IFN system by blocking IFN synthesis, by inhibiting IFN signaling or by inhibiting the functions of IFN-induced proteins such as dsRNA-dependent protein kinase (PKR). The most important signal transduction pathway for IFNs is the Jak-STAT pathway[3]. IFNα and IFNβ activate STAT1, STAT2 and often STAT3. Signal transducers and activators of transcription (STAT) proteins are activated by members of the Jak kinase family through the phosphorylation of a single tyrosine residue[4]. Activated STATs form dimers, translocate into the nucleus and bind specific DNA elements in the promoters of target genes[5, 6]. This activation cycle is terminated by tyrosine dephosphorylation in the nucleus, followed by the decay of dimers and the nuclear export of STATs[7, 8]. Negative regulators of this signal transduction pathway have been found at three levels. First, the suppressor of cytokine signaling (SOCS) family members SOCS1 and SOCS3 prevent phosphorylation and activation of IFN induced STATs by inhibiting the IFN receptor associated Jak kinases[9]. Second, downstream of STAT activation by tyrosine phosphorylation, IFN induced gene transcription can be inhibited by protein inhibitor of activated STAT1 (PIAS1). PIAS1 inhibits binding of STAT1 dimers to the response elements in the promoters of target genes[10]. The binding of PIAS1 to STAT1 is regulated by methylation of STAT1 by protein arginine methyl-transferase PRMT1[11]. Arginine methylation inhibits binding of PIAS1 to STAT1, whereas demethylation of STAT1 enhances its association with PIAS1. A third step of negative regulation occurs through dephosphorylation and deactivation of STATs in the nucleus. Recently, the 45 kDa isoform of protein tyrosine phosphatase TC-PTP was identified as the nuclear STAT1 phosphatase[12].

The inventors (hereinafter sometimes referred to as "we") have shown previously that the expression of HCV proteins in human osteosarcoma cell lines inhibits IFNα

induced signaling through the Jak-STAT pathway[13]. The inventors confirmed these findings also in livers of transgenic mice expressing the entire HCV open reading frame under the control of an alpha1-antitrypsin gene promoter[14]. Both in the cell lines and in the mouse livers, activation of STATs by tyrosine phosphorylation was not inhibited, but binding of activated STAT1, STAT2 and STAT3 to DNA was strongly reduced. The inventors hypothesized that HCV proteins may induce cellular proteins that inhibit STAT DNA binding, for instance PIAS1 or other yet unknown proteins, or that one or more nuclear phosphatases could be induced, resulting in accelerated dephosphorylation and decay of STAT dimers. Since we could not detect an induction of PIAS1 by HCV proteins neither in the cell lines nor in the transgenic mouse livers, we screened mouse liver extracts for an overexpressed phosphatase.

The inventors have found that the catalytic subunit of protein phosphatase 2A (PP2Ac) was overexpressed in liver extracts of HCV transgenic mice. Interestingly, expression of an N-terminally modified catalytic subunit of PP2A in the human hepatoma cell line Huh7 resulted in hypomethylation of STAT1 leading to inhibition of STAT1-DNA binding through increased binding to PIAS1. The relevance of these findings for the human disease was confirmed in liver biopsies from patients with chronic hepatitis C.

Moreover, the inventors have found that treatment with a methyl group donor can restore IFN signaling. S-adenosyl-L-methionine (SAMe, also called AdoMet or S-Adenosylmethionine) is a methyl group donor produced naturally in all living organisms. Under normal circumstances, it is produced in the cells by the transfer of an adenosyl group (derived from ATP) to methionine by the enzyme methionine adenosyl transferase. After enzymatic transfer of its methyl group, SAMe is metabolized to homocysteine, a potential toxic substance. Homocysteine can by recycled to SAMe by transfer of a methyl group from betaine by betaine-homocysteine methyltransferase (BHMT). By using a combination of SAMe and betaine, the inventors found a significant increase in the induction of interferon stimulated genes by IFNα.

The inventors have found that treatment of cultured cells with SAMe increases the methylation of STAT1 and increases IFN signaling. Since methylated STAT1 can not be bound by its inhibitor PIAS1, it is a better IFN signal transducer.

Currently, patients with chronic hepatitis C are treated with pegylated interferon alpha 2a or 2b in combination with ribavirin. 50-60% of the patients treated with pegylated interferon and ribavirin do not respond to the treatment. For patients with genotype 1, the treatment success rate is 40-45%. The work of the inventors indicates that correction of the methylation state of STAT1 should increase the responsiveness of patients to therapy for hepatitis C. Although others have suggested treatment of patients with a combination of interferon alpha, ribavirin, and antioxidants including SAMe (see WO 03/024461), they do not appreciate the importance of restoring STAT1 methylation, and they use SAMe as an antioxidant to reduce ribavirin-related hemolysis.

Further objects, features and advantages of the present invention will become apparent from the Detailed Description of Preferred Embodiments, which follows, when considered in connection with the attached Drawings.

SUMMARY OF THE INVENTION

It is a general object of the invention to increase the level of STAT1 methylation in vivo.

A first preferred embodiment of the invention is a compound increasing methylation of a STAT1 in vivo.

A second preferred embodiment is a compound increasing methylation of a STAT1 in vivo wherein the compound acts by increasing or decreasing an activity of a protein selected from the group consisting of: PP2A2, PRMT1, and PIAS1.

A further preferred embodiment is a therapeutic composition comprising the compound of second embodiment and a pharmacologically-acceptable carrier.

A third preferred embodiment is a compound decreasing activity of a PP2A.

Another preferred embodiment is a therapeutic composition comprising a compound of the third embodiment and a pharmacologically-acceptable carrier.

A fourth preferred embodiment is a method of selecting a drug to treat hepatitis C, comprising the steps of: providing a plurality of drug candidates, assaying the candidates to ascertain a change in a level of STAT1 methylation upon application of the candidates to a model system, selecting candidates that increase methylation of STAT1, and selecting a candidate from among those selected candidates that is effective in treating hepatitis C.

A fifth preferred embodiment is the fourth embodiment, wherein said model system is selected from the group consisting of a protein binding assay, a protein activity assay, a cell culture model, and an animal model.

A sixth preferred embodiment is the fifth embodiment wherein the last-named step comprises testing the drug in an animal model of hepatitis C infection.

Another preferred embodiment is a drug developed by the method of the sixth embodiment.

A seventh preferred embodiment is a method if selecting a drug to treat hepatitis C, comprising the steps of providing a plurality of drug candidates, assaying the candidates to ascertain a change in a level of PP2A activity upon application of the candidates to a model system, selecting candidates that change the level of PP2A activity, and selecting a candidate from among those selected candidates that are effective in treating hepatitis C.

Yet another preferred embodiment is the seventh embodiment wherein the model system is selected from the group consisting of a protein binding assay, a protein activity assay, a cell culture model, and an animal model.

Still another preferred embodiment is the seventh embodiment wherein the last-named step comprises testing the drug in an animal model of hepatitis C infection.

Yet another preferred embodiment is a drug selected by the method of the seventh embodiment.

An eighth preferred embodiment is a method of treating hepatitis C infection, comprising enhancing a level of methylation of STAT1.

A ninth preferred embodiment is the method of the eighth embodiment, further comprising increasing or decreasing an activity of a protein selected from the group consisting of: PPA2, PRMT1, and PIAS1.

Still another preferred embodiment is the method of the ninth embodiment, wherein said increasing or decreasing step is carried out by administering a therapeutic composition.

A tenth preferred embodiment of the invention is a method of treating hepatitis C infection, comprising reducing an activity of PP2A.

Yet another preferred embodiment of the invention is the method of the tenth embodiment, wherein said reducing is carried out by administering a therapeutic composition.

Still another preferred embodiment of the invention is the method of the eighth embodiment further comprising the step of administering a drug selected by the method of the fourth embodiment.

Yet another preferred embodiment of the invention is the method of the eighth embodiment further comprising the step of administering a drug selected by the method of the seventh embodiment.

Still another preferred embodiment is the use of methyl group donor for the manufacture of a pharmaceutical composition, wherein the pharmaceutical composition comprises a methyl-group donor in an amount effective to increase a level of STAT1 methylation in a patient.

An eleventh another preferred embodiment is the use of a methyl group donor to increase a level of STAT1 methylation in a patient, comprising (1) selecting a patient, and (2) treating the patient with a methyl group donor in an amount effective to increase the level of STAT1 methylation in the patient.

Yet another preferred embodiment is the use of the eleventh embodiment, further comprising the step of monitoring said level of STAT1 methylation in said patient.

Still another preferred embodiment is a pharmaceutical composition consisting essentially of interferon, ribavarin, a methyl group donor, and a pharmacologically-acceptable carrier.

Yet another preferred embodiment is a pharmaceutical composition consisting essentially of interferon, ribavarin, a methyl group donor, and a pharmacologically-acceptable carrier.

Still another preferred embodiment is any of the previously-named embodiments with a methyl group donor, wherein the methyl group donor is SAMe.

Yet another preferred embodiment is any of the previously-named embodiments with a methyl group donor, wherein betaine is used in conjunction with said methyl group donor.

A twelfth preferred embodiment is a method of monitoring a patient, comprising the steps of (1) selecting a patient undergoing treatment for viral infection, and (2) testing one or more samples from the patient to ascertain a level of STAT1 methylation.

Still another preferred embodiment is the method of the twelfth embodiment, wherein the viral infection is a hepatitis C infection.

Yet another preferred embodiment is a pharmaceutical composition comprising pegylated interferon, ribavarin, SAMe, betaine, and a pharmacologically-acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Abbreviations as used herein are:
DMA, dimethylarginine; EMSA, electrophoretic mobility shift assay; HA-PP2Ac, catalytic subunit of PP2A fused to an influenza hemagglutinin derived nine-amino-acid epitope tag; HCV, hepatitis C virus; hIFNα (hIFNa), human interferon alpha; ISRE, interferon stimulated response element; MTA, 5'-methyl-thioadenosine; OA, Okadaic Acid; PIAS1, protein inhibitor of activated STAT1; PKR, dsRNA-dependent protein kinase; PP2A, protein phosphatase 2A; PP2Ac, catalytic subunit of PP2A; PRMT1, protein arginine methyl-transferase 1; SOCS, suppressor of cytokine signaling; SAMe, S-adenosylmethionine; STAT, signal transducer and activator of transcription; TC-PTP, T cell protein tyrosine phosphatase.

Figure 1. TC-PTP, STAT1 phosphorylation, and PP2A expression in HCV transgenic mice.

(A) Nuclear tyrosine phosphatase TC-PTP is not upregulated in B6HCV mice. Liver extracts of untreated or IFNα (1000U/g of body weight) treated C57BL6 and B6HCV were tested for TC-PTP expression by Western blot. Blots were stripped and reprobed for actin as loading control. No difference in TC-PTP expression was detected.
(B) Overexpression of PP2Ac in transgenic mice. Western blot with whole cell extracts from C57BL6 and B6HCV mouse livers show increased amounts of PP2Ac. The membrane was stripped and reprobed with actin antibodies as a loading control. Blots were quantified using NIH Image software and values represent the mean ± SEM of 14 mice in each group. *** Indicates a statistical significant difference ($P < 0.001$) between B6HCV and C57BL6 groups (ANOVA test).
(C) PP2Ac activity measurements. Serine/threonine phosphatase activity of PP2Ac from whole cell extracts was performed using Serine/Threonine Phosphatase Assay System (Promega) according to the manufacturer's instructions. Shown are the mean values ± SEM of 2 animals in each group. The repetition of the experiment gave the same result (not shown). * indicates a statistical significant difference ($P < 0.05$) between B6HCV and C57BL6 mice (ANOVA test).
(D) Phospho-serine and phospho-tyrosine status of STAT1 is not impaired in HCV transgenic mice. C57BL6 and B6HCV mice were left untreated or treated with mIFNα (1000U/g of body weight, 20 min). Liver extracts were probed for tyrosine phosphorylation of STAT1 by Western blot with STAT1 phosphotyrosine specific antibodies (PY-STAT1) and for serine phosphorylation by a STAT1 immunoprecipitation followed by Western blot with STAT1 phosphoserine specific antibodies (PS-STAT1). Blots were stripped and reprobed with STAT1 antibodies. No difference between C57BL6 and B6HCV was detected.

Figure 2. Expression of an epitope-tagged phosphatase 2A catalytic subunit inhibits IFNα induced Jak-STAT signaling.
(A) From eight Huh7 derived clones with stable expression of hemagglutinin (HA) tagged PP2Ac the two clones with the highest expression levels were used for analysis of IFNα induced signal transduction. Shown are the results with one of them (the same results were found with the other clone). Lysates from unstimulated Huh7 and HA-PP2Ac expressing cells were analyzed for the expression of endogenous PP2Ac and transfected HA-PP2Ac by Western blot with PP2Ac specific antibodies. Membranes were then stripped and reprobed with HA antibodies to confirm the presence of the HA-tag and with actin antibodies as a loading control.
(B) Epitope-tagged PP2Ac allows expression of functionally active phosphatase 2A catalytic subunit. Serine/threonine phosphatase activity of PP2Ac from whole cell lysates was measured using the Serine/Threonine Phosphatase Assay System (Promega) according to the manufacturer's instructions. Values are the mean ± SEM of 2 independent experiments.
(C) Expression of HA-PP2Ac impairs Jak-STAT signaling. Huh7 and HA-PP2Ac expressing cells were incubated in the presence or in the absence of hIFNα (1000U/ml, 20 min) with or without pretreatment with okadaic acid (100nM, 60 min) prior to hIFNα stimulation as indicated. Nuclear extracts were analyzed with EMSAs using the SIE-m67 oligonucleotide probe.
(D) Serine and tyrosine phosphorylation of STAT1 is not impaired by HA-PP2Ac expression. Whole cell lysates from unstimulated or stimulated cells were probed for tyrosine phosphorylation of STAT1 (PY-STAT1) or serine phosphorylation (PS-STAT1) with specific antibodies by Western blot or STAT1 immunoprecipitation / Western blot, respectively. Blots were stripped and reprobed with STAT1 antibodies.

(E) Incubation of purified, active PP2Ac with nuclear extracts from Huh7 cells does not impair Jak-STAT signaling. Nuclear extracts from unstimulated or hIFNα (1000U/ml, 20 min) treated cells were incubated with purified PP2A for 10 or 20 min as indicated (samples in duplicates). EMSA was performed with the SIE-m67 oligonucleotide probe.

Figure 3. PP2Ac overexpression reduces the arginine methylation of STAT1 resulting in enhanced binding of PIAS1 to STAT1.
(A) Expression of HA-PP2Ac reduces STAT1 arginine methylation. Whole cell lysates from Huh7 and HA-PP2Ac were subject to immunoprecipitation with mono/dimethyl arginine antibodies. Resolved proteins were immunoblotted for STAT1.
(B) Reduced arginine methylation of STAT1 in liver extracts of HCV transgenic mice. Liver cell extracts of C57BL6 and B6HCV mice were immunoprecipitated with DMA antibodies. The amount of precipitated STAT1 was analyzed by Western blot with STAT1 specific antibodies.
(C) Semiquantitative determination of STAT1 methylation. Liver extracts of untreated mice were immunoprecipitated with DMA antibodies. The amount of precipitated STAT1 was analyzed by Western blot with STAT1 specific antibodies. The signal intensities were quantified with the NIH image computer program. The bar diagram shows the mean values (plus standard errors) of 4 C57BL76 and 4 B6HCV mice.
(D) Increased binding of PIAS1 to STAT1 in cells expressing HA-PP2Ac. Whole cell lysates from Huh7 and HA-PP2Ac cells were subject to immunoprecipitation with STAT1 antibodies and then blotted for PIAS1. As a positive control, Huh7 cells were treated with for 1 hour with 0.3 mM MTA.
(E) Increased binding of PIAS1 to STAT1 in liver extracts from HCV transgenic mice. Liver extracts from C57BL6 and B6HCV mouse were subject to immunoprecipitation with STAT1 antibodies and then blotted for PIAS1.
(F) Inhibition of PP2Ac with okadaic acid increases arginine methylation of STAT1. Huh7 cells and HA-PP2Ac expressing cells were treated with 1000 U/ml hIFNα for 20 minutes after preincubation with or without okadaic acid (100nM, 60 min). The reduction of arginine methylation observed in cells expressing HA-PP2Ac in the absence of okadaic acid (lane 2) is partially reversed by pretreatment with 100nM okadaic acid (lane 3). The lower panel shows the PP2Ac phosphatase activity in the corresponding samples. The activity is increased in untreated HA-PP2Ac expressing cells compared to Huh7 cells, but strongly inhibited by pretreatment with okadaic acid. Serine/threonine phosphatase activity of PP2Ac from whole cell lysates was measured using the Serine/Threonine Phosphatase Assay System (Promega) according to the manufacturer's instructions.

Figure 4. Impaired IFNα induced Jak-STAT signaling in liver biopsies of patients with chronic hepatitis C.
(A) Human liver biopsies can be stimulated *in vitro* with IFNs. 5-10 mm long tissue cylinders obtained by percutaneous liver biopsy with a 1.4mm thick biopsy needle were incubated immediately after retrieval in phosphate buffered saline (PBS) alone or in PBS with 1000U/ml human IFNα for the indicated length of time. Cryosections (5 μm) were made and subcellular localization of PY-STAT1 was determined using an antibody against Tyr-701 of STAT1. Nuclei were stained with Hoechst stain. The white size bars indicate 20 μm. Activation of STAT1 and nuclear translocation was uniform throughout the biopsy cylinder. Maximum activation of STAT1 occurred after 20 min.
(B) Quantification of activated STAT1 in liver cells nuclei. Shown are values (percent liver cell nuclei with positive staining for PY-STAT1) from 4 patients: Number 53 had a nodular regenerative hyperplasia without liver fibrosis, number 59 had a chronic hepatitis

C with a liver cirrhosis, number 60 had a chronic hepatitis C with mild fibrosis, and number 61 was recovered from hepatitis B and showed no fibrosis on routine histology. For each time point and patient, a minimum of 200 cells was counted.
(C) Semiquantitative assessment of EMSAs with nuclear extracts from liver biopsies of patients with chronic hepatitis C or controls. Liver biopsies were incubated in the absence or in the presence of hIFNα (1000U/ml) for 20 minutes and nuclear extracts were prepared. EMSAs were performed using 10 µl of nuclear extracts with SIE-m67 oligonucleotide probe. Shown are representative samples: Patients number 64, 83 and 104 were controls, patients number 69, 111, 115, 78 and 73 had chronic hepatitis C. An example of signal quantification is shown with patient number 73. The autoradiogram signal intensity was measured using NIH Image software and then normalized to 1 µg of total protein used for EMSA. The response to hIFNα stimulation is calculated by a ratio of the normalized signal intensity after hIFNα treatment divided by the normalized signal intensity of PBS controls (= fold increase).
(D) Impaired IFNα induced signaling in liver biopsies from patients with chronic hepatitis C. Shown are box-plots of the fold increase in EMSA signals after IFNα treatment for 25 patients with chronic hepatitis C and 10 controls. The difference between the groups was analyzed with the Mann-Whitney U test and found to be significant at $p = 0.03$.

Figure 5. Expression, phosphorylation and arginine methylation of STAT1, and expression of PP2Ac in human liver cells.
(A) Tyrosine phosphorylation of STAT1 is not impaired by the presence of the HCV. Biopsies from controls (patients number 64, 83, 89) and from patients with chronic hepatitis C (patients number 69, 119, 132) were incubated with hIFNα (1000U/ml, 20 min) and extracts were probed for PY-STAT1 to check the phosphorylation status of STAT1. The two bands seen in some samples are the larger STAT1α and the shorter STAT1β, respectively. Nitrocellulose membranes were then stained with Blot-FastStain to verify the loading amount of total protein. One of the stained proteins (180 kDa in size) is shown as an example.
(B) STAT1 is overexpressed in liver cells of HCV patients. Biopsy extracts from healthy (numbers 83, 143, 64) and HCV patients (numbers 81,82,87) were probed for STAT1 to determine the expression level. Nitrocellulose membranes were then stained with Blot-FastStain to ensure equal protein loading. One of the proteins (180 kDa in size) is shown.
(C) Semiquantitative assessment of STAT1 expression. The STAT1 signal intensity in Western blots was quantified with NIH Image software. The results (arbitrary units) are shown in a box plot diagram. The difference between controls and HCV patients is statistically significant ($P < 0.0001$, Mann-Whitney U test).
(D) Overexpression of PP2Ac in liver cells of HCV patients. Liver biopsy extracts from controls (numbers 83, 143, 64) and HCV patients (numbers 81, 82, 87) were analyzed with Western blot using a PP2Ac specific antibody. The same 180 kDa protein as above is shown as a loading control.
(E) Semiquantitative assessment of PP2Ac expression. The PP2Ac signal intensity in Western blots was quantified with NIH Image software. The results (arbitrary units) are shown in a box plot diagram. The difference is significant at $p = 0.04$ (Mann-Whitney U test).
(F) STAT1 is hypomethylated in HCV patients. Whole cell lysates from unstimulated healthy (numbers 153, 154) and HCV patients (numbers 132, 138) were subject to immunoprecipitation with DMA antibodies and then blotted for STAT1. The two bands seen in some samples are STAT1α and STAT1β, respectively.

(G) Quantification of STAT1 methylation. The STAT1 specific signal intensities after immunoprecipitation with DMA antibodies (upper panel of figure 5F) were divided through the STAT1 specific signal intensities in a Western blot (not shown) for two controls (numbers 153, 154) and two patients with chronic hepatitis C (numbers 132, 138). STAT1 is hypomethylated in extracts from liver biopsies of patients with chronic hepatitis C as compared to controls.
(H) Increased binding of PIAS1 to STAT1 in HCV patients. Whole cell extracts from unstimulated control patients (number 148, 154) and HCV patients (numbers 132, 138) were subject to immunoprecipitation with STAT1 antibodies and resolved proteins were blotted for PIAS1.
(I) Nuclear tyrosine phosphatase TC-PTP is equally expressed in human liver cells of healthy and HCV patients. Whole cell lysates from unstimulated liver biopsies were probed for TC-PTP to determine the expression level. Nitrocellulose membranes were then stained with Blot-FastStain to verify the loading amount of total protein.

Figure 6. STAT1 expression, PP2Ac expression and EMSA signal intensity of STAT1 are independent of inflammation and fibrosis of liver biopsies.
Pathological grading and staging of liver biopsies was according to Hytiroglou et al.[16]: Grade of inflammation: 0 = no inflammation, 1 = mild, 2 = moderate, 3= severe; Stage of fibrosis: 0 = no fibrosis, 1 = mild, 2 = moderate, 3 = severe, 4 = cirrhosis. Biopsies were grouped in 4 categories of inflammation (left column) or 5 categories of fibrosis (right column). Shown are box plot diagrams of protein expression levels of STAT1 (first row) and PP2Ac (second row) and of STAT1 gel shift signal intensities (third row). None of the differences between groups were statistically significant when tested with the parametric one-factor ANOVA test or the non-parametric Kruskal-Wallis test by ranks.

Figure 7. Treatment to restore STAT1 methylation and interferon signaling. (A) EMSA with m67 oligonucleotide showing that treatment with AdoMet (SAMe) restores IFN signaling in cells expressing HA-PP2Ac. (B) Immunoprecipitation analysis shows that AdoMet (SAMe) restores STAT1 methylation in cells expressing HA-PP2Ac and reduces association of STAT1 with the inhibitor Pias1.

Figure 8. Treatment of Huh7 and HA-PP2A cells with Adomet and Betaine increases the induction of the IP-10 gene by IFNα. The cells were treated overnight with AdoMet and/or Betaine using the concentrations indicated on the graph. They were ten treated again with AdoMet, Betaine, and IFNα for 6 hours. After extraction of RNA, real time RT-PCR was performed to quantify the amount of IP-10 mRNA.

DETAILED DESCRIPTION OF THE INVENTION

Signaling through the Jak-STAT pathway is crucial for most of the known effects of both type I and type II interferons. In cell lines and in transgenic mice the inventors have previously found inhibition of IFNα induced binding of activated STATs to their cognate response elements by HCV proteins. Interestingly, in none of these cases any of the well-known Jak-STAT inhibitors SOCS1, SOCS3 or PIAS1 were induced. The inventors have now found that hypomethylation of STAT1 is the basis for the observed inhibition of Jak-STAT signaling. Methylation of STAT1 has recently been recognized as a third important posttranslational modification besides tyrosine and serine phosphorylation that regulates STAT mediated transcriptional activation of target genes[11]. Methylation of of STAT1 is catalyzed by PRMT1, and inhibits the binding of PIAS1 to STAT1[11]. Binding of PRMT1

to the IFNα receptor has been reported before[27]. The same authors observed impairment of the antiviral and antiproliferative effects of IFNβ in HeLa cells when the expression of PRMT1 was reduced by antisense cDNA[27]. Interestingly, methyl-thioadenosine (MTA) is an inhibitor of PRMT1 that accumulates in many transformed cells. Through inhibition of STAT1 methylation, MTA might contribute to the relative unresponsiveness of many cancer cells towards the antiproliferative effects of IFNs[11]. The inventors have found for the first time that a virus, HCV, can induce IFNα hyporesponsiveness by blocking methylation of STAT1. The expression of HCV proteins in liver cells of transgenic mice reduced STAT1 methylation and consequently increased association of STAT1 with PIAS1. Importantly, reduced methylation and increased PIAS1-STAT1 binding was also observed in liver biopsies from patients with chronic hepatitis C.

HCV proteins may directly inhibit PRMT1 or stimulate demethylation by an as yet unknown enzyme. However, the inventors have found that PP2A is involved in mediating this effect: First, PP2A was found to be overexpressed in HCV transgenic mice and in liver biopsies from patients with chronic hepatitis C. Second, Huh7 cell stably expressing the catalytically active HA-PP2Ac had hypomethylated STAT1 in the absence of any HCV proteins. Third, inhibition of PP2A with okadaic acid partially restored methylation of STAT1.

Alternatively, PP2A might influence DNA binding of STAT1 directly by changing the phosphorylation status of STAT1, without having any influence on the methylation status of STAT1. For example, PP2A has been reported to inhibit DNA binding of STAT1 by dephosphorylation of serine 727 without affecting tyrosine phosphorylation in an *in vitro* assay with extracts from U937 cells[26]. In human T cells or lymphoma cells, inhibition of PP2A was found to induce serine phosphorylation of STAT3, inhibit tyrosine phosphorylation of STAT3, and to induce relocation of STAT3 from the nucleus to the cytoplasm[23]. Furthermore, PP2A inhibition by okadaic acid was found to strongly enhance IL-3 induced tyrosine phosphorylation of STAT5 in 32Dcl3 myeloid progenitor cells[24]. While these various experimental systems yield different results with regard to the influence of PP2A on Jak-STAT signal transduction, they nevertheless raise the possibility that PP2Ac upregulation in HCV transgenic mice and in liver cells of patients with chronic hepatitis C may influence STAT function by altering tyrosine or serine phosphorylation of STAT1. The inventors therefore looked carefully for changes of serine or tyrosine phosphorylation caused by overexpression of PP2A, but did not detect significant differences in extracts from HA-PP2Ac expressing cells compared with Huh7 cells nor in liver extracts from HCV transgenic mice compared with control mice. The inventors conclude that in our experimental systems, the influence of PP2A is indirect through the inhibition of methylation or the stimulation of demethylation of STAT1. So far, we found no good evidence for a direct interaction between PP2A and PRMT1, and we can not exclude that the effect of PP2A is mediated through other proteins. However, the fact that expression of HA-PP2Ac in Huh7 cells caused a hypomethylation of STAT1 and inhibitors of PP2A restored methylation indicates that PP2Ac is upstream of PRMT1. Interestingly, the PP2A holoenzyme assembly itself is regulated by reversible methylation of the catalytic subunit PP2Ac. However, the methyltransferase involved is PPMT and not PRMT1[28, 29].

PP2A is involved in a wide range of cellular processes including cell cycle regulation, cell morphology, development, signal transduction, translation, apoptosis and stress response[21]. A number of viral proteins have been found to interact with subunits of

PP2A, amongst them polyoma small t and middle T, SV40 small t, the HIV protein vpr and the adenovirus E4orf4 protein. These associations alter the function of PP2A without affecting its expression level. The expression of PP2Ac is controlled by an autoregulatory translational mechanism[22]. However, downregulation of PP2Ac has been found, for instance during all-*trans*-retinoic acid-induced differentiation of HL-60 cells[30] or during peroxisome proliferator-activated receptor-γ induced adipocyte differentiation[31]. Likewise, upregulation of PP2Ac was found in macrophages in response to colony-stimulating factor 1[32]. Here we report for the first time that expression of viral proteins can upregulate PP2Ac. Given the central role of PP2A in the regulation of cellular homeostasis, it is likely that this upregulation has many additional effects on cells infected with HCV. However, IFN signaling was found to be an important target for viral interference with host defense, and inhibition of STAT1 might well be a crucial effect of PP2Ac upregulation by HCV.

What cells in the liver are crucial for the observed interference of HCV with IFNα signaling? In human liver biopsies (Figure 4A), not only hepatocytes, but also non-parenchymal resident liver cells and infiltrating lymphocytes have nuclear localized phospho-STAT1, and all of them could be the site of PP2Ac upregulation and inhibition of STAT1 signaling. However, the inventors have found that the HCV transgenic mice have no inflammatory infiltrates[14], but still show increased PP2Ac expression levels and impaired STAT1 signaling. Furthermore, the inventors have found no correlation was found between the degree of inflammation in liver biopsies and the amount of STAT1, PP2Ac or the strength of STAT1 DNA binding (Figure 6). Therefore, the most likely site of HCV interference with IFNα signaling is the hepatocyte.

HCV has a striking capability to establish a chronic infection, and attenuation of IFNα signaling is an important advantage of the virus early in the course of the infection. However, HCV can not block IFNα signaling completely, as demonstrated by the success of therapies based on the application of pharmacological doses of recombinant IFNα in some but not all patients with chronic hepatitis C. It is not well understood, which host factors determine the response to IFNα therapies. PP2Ac expression levels and the strength of IFNα induced signaling through the Jak-STAT pathway might represent such host factors. The mean PP2Ac expression level of the 8 patients that were not cured by standard therapies with pegylated IFN-α and ribavirin is 5812 (arbitrary units), compared to 590 for the patients with a sustained response. Likewise, the mean fold increase in EMSA signals after ex vivo IFNα treatment of biopsies is 3.3 in therapy responders, and 2.3 in non responders (table 1). However, the low sample size in this study does not allow a statistical analysis, and we conclude that we do not know if patients with high expression levels of PP2Ac are less responsive to IFNα treatments. Presently, we can only speculate that interference of HCV with IFNα signaling might also impair the success of IFNα therapies. Should further studies find that increased PP2Ac levels impair the response to IFNα based treatments, then treatment strategies aimed at reversing the inhibitory effect of PP2Ac on IFNα signaling might enhance the response rates to current standard therapies with IFNα and ribavirin.

We describe here that HCV interferes with IFNα signaling via upregulation of PP2Ac. As a consequence, STAT1 methylation is reduced. Because hypomethylated STAT1 is bound by its inhibitor PIAS1, the transcriptional activation of IFN target genes is impaired. The inhibition of the IFNα induced antiviral response might allow HCV to evade this important first line of defense of the host and to establish a chronic infection.

Restoring STAT1 methylation in cells in culture restores IFN signaling, thus treatment of patients receiving therapy for HCV infection with an agent to restore STAT1 methylation should improve the efficacy of therapy. One example of such an agent is SAMe. Betaine used in conjunction with SAMe potentiates the effects of SAMe on STAT1 methylation and interferon signaling. After transfer of the methylgroup, SAMe is metabolized to homocysteine, a potential toxic metabolite. Homocysteine can be recycled to SAMe using betaine as a methyl group donor by the enzyme betaine-homocysteine-methyltransferase (BHMT). The combined use of SAMe and betaine significantly improves the methylation of STAT1 and the induction of interferon stimulated genes by IFNα. Another way to achieve the therapeutic goal of increased STAT1 methylation would be to decrease activity of PP2A.

Methods as used in the examples

Patients, Biopsies

From February, 2001, to April, 2002, all patients with chronic hepatitis C referred to the outpatient liver clinic of the University Hospital Basel were asked for their permission to use part of the liver biopsy for this study. The protocol was approved by the ethical commission of Basel. Written informed consent was obtained from all patients that agreed to participate in the study. After removal of a 20 to 25mm long biopsy specimen for routine histopathological workup for grading and staging of the liver disease according to Hytiroglou et al.[16], the remaining 5 to 20 mm long biopsy cylinders were used for the preparation of cytoplasmic and nuclear extracts and in some cases for immunofluorescence studies. For EMSA and Western blots, all samples that yielded at least 0.5 micrograms per microliter were used. The other samples were discarded (about 30-40% of all biopsies obtained). For non-HCV controls, patients that underwent ultrasound-guided liver biopsies of focal lesions (mostly metastasis of carcinomas) were asked for their permission to obtain a biopsy from the normal liver tissue outside the focal lesion. Written informed consent was obtained. Again, a part of the biopsy was used for routine histopathological diagnosis, and the remaining tissue for the preparation of cell extracts as described below. Only samples with confirmed absence of liver disease in the routine histopathological workup were used as controls in the present study.

Cell culture, Transfection

Cells were cultured in 10% calf serum/Minimum Essential Medium (Invitrogen) HA-PP2Ac cell lines were obtained by stably transfecting Huh7 cells in 6 cm tissue culture dishes using Effectene Transfection Reagent kit (Qiagen) according to the manufacturer's instructions. After 48 hours, the cells were rinsed with PBS and incubated with fresh medium (10% calf serum/Minimum Essential Medium) supplemented with penicillin, streptomycin and 800 µg/ml G418. Individual colonies were picked 7 to 10 days later and propagated. In eight clones out of 20 that were analyzed for HA-PP2Ac expression the transgene was detected by anti-HA Western blot. The two clones with the highest expression level were used for the analysis of IFNα induced intracellular signaling.

Preparation of extracts from cells

Cells were lysed on the plates in 200 µl of lysis buffer containing 100 mM NaCl, 50 mM TRIS pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 1 mM PMSF, and 1

mM vanadate. Lysates were centrifuged at 14,000 rpm for 5 min and protein concentration was determined by Lowry (BioRad Protein Assay).

For mouse and human whole cell extracts, samples were dounce homogenized in 500 µl or 120 µl of lysis buffer respectively.

For cell nuclear extracts, cells were rinsed with cold PBS, scraped from plate and lysed in Low Salt Buffer containing 200 mM Hepes pH 7.6, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.2% NP-40, 10% glycerol and 0.1 mM vanadate. Then they were centrifuged at 15,000 rpm for 5 min and the pellet was resuspended in High Salt Buffer containing Low Salt Buffer supplemented with 420 mM NaCl. After centrifugation, aliquots were made for EMSA.

Immunofluorescence with human liver biopsies

For liver biopsy immunostaining, biopsies were performed with a Menghini liver biopsy set (Sterylab, Rho, Italy). Parts of the biopsy were incubated in PBS or PBS with 1000 U/ml IFNα at 37°C for different periods of time. Cryosections (5 µm) were made onto glass slides. Slices were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized in 0.1% Triton X-100 for 2 hours and blocked in 3% BSA-0.1% Triton X-100 for 1 hour. Then they were washed with TBST prior to incubation with anti-PY-STAT1 (Cell Signalling) overnight at 37°C. After three washes with TBST, they were incubated with Cy3 conjugated secondary antibody (Amersham) for 1h30 hour at room temperature. Nuclear staining was performed with Hoechst (Amersham) for 5 min at room temperature. After washing, a coverslip was mounted in FluorSave Reagent (Calbiochem).

For quantification of nuclear phospho-STAT1 staining, a minimum of 100 cells was counted under fluorescent microscope for each sample.

Preparation of extracts from human liver biopsies

For human biopsy cytoplasmic/nuclear extracts, samples were homogenized in 120 µl of Buffer A containing 20 mM Hepes pH 7.6, 10 mM NaCl, 3 mM MgCl2, 0.1% NP-40, 10% glycerol, 0.2 mM EDTA and 1 mM DTT. After centrifugation at 2000 rpm for 5 min, supernatant (cytoplasmic extracts) was removed and the pellet was washed with 200 µl of Buffer B containing 20 mM Hepes pH 7.6, 0.2 mM EDTA, 20% glycerol and 1 mM DTT, and then centrifuged at 3000 rpm for 5 min. The pellet was then resuspended in 60 µl of Buffer C containing 20 mM Hepes pH 7.6, 400 mM NaCl, 0.2% EDTA, 20% glycerol and 1mM DTT. After centrifugation, the supernatant (nuclear extracts) was taken and 10 µl aliquots were made for EMSA.

Immunoprecipitation and immunoblotting

Cell, mouse liver and human liver lysates were incubated with anti-PP2A (Upstate), anti-STAT1 (Transduction Laboratories), anti-TC-PTP, anti-Actin (Santa Cruz) or monoclonal mono/dimethyl-arginine (Abcam) antibodies for overnight at 4°C. Then they were added to protein A-Sepharose (Sigma) for 3 hours at 4°C. After SDS-PAGE and transfer onto nitrocellulose membrane (Schleicher & Schuell), proteins were detected with

anti-STAT1 or anti-PIAS1 antibodies. Blots were analyzed by densitometry using NIH image software.

For loading amount control of human liver biopsy samples, nitrocellulose membrane was stained using Blot-FastStain (GenoTech) according to the manufacturer's instruction.

Electrophoretic mobility shift assays

EMSAs were performed using 2 μl nuclear extract aliquots for cells and 10 μl aliquots for human biopsies, and end-labeled oligonucleotide SIE-m67 corresponding to STAT response element sequences[13].

PP2A phosphatase activity assay

PP2A activity was performed with whole cell extracts or nuclear extracts using Serine/Threonine Phosphatase Assay System (Promega) according to the manufacturer's instructions, in PPTase-2A buffer containing 250 mM imidazole pH 7.2, 1 mM EGTA, 0.1% β-mercaptoethanol and 0.5 mg/ml BSA.

Example 1: PP2Ac overexpression in HCV transgenic mice

Previously, we reported inhibition of IFNα induced signaling through the Jak-STAT pathway by HCV proteins in cell lines and in livers of transgenic mice[13, 14]. In both experimental systems, interference with IFNα signaling occurred at the level of STAT-DNA interactions, and could be detected by electrophoretic mobility shift assays (EMSA) with nuclear extracts and radiolabeled DNA oligonucleotide containing the interferon stimulated response element (ISRE) or a mutated serum inducible element (SIE) designated SIE-m67. The activation of STAT proteins through tyrosine phosphorylation at the receptor kinase complex was not inhibited. Furthermore, we could not detect a block in the nuclear import of activated STATs. We therefore concluded that the interference of HCV proteins with the Jak-STAT signaling occurs in the nucleus, either through inhibition of STAT-DNA binding, or through an increased rate of STAT dephosphorylation by one or more phosphatases.

Recently, TC-PTP was identified as the elusive nuclear STAT1 protein tyrosine phosphatase[12]. TC-PTP might be upregulated by viral proteins and could increase the dephosphorylation rate of phospho-STAT1 in the nucleus. However, the amount of TC-PTP in liver cell extracts of transgenic mice was the same as in control mice (Figure 1A). A review of the literature drew our attention to protein phosphatase 2A (PP2A). PP2A is a heterotrimeric protein phosphatase consisting of a 36 kDa catalytic C subunit (PP2Ac), a 65 kDa structural A subunit and a variable regulatory B subunit. PP2A is primarily a serine/threonine phosphatase, but under certain conditions it has an independent phosphotyrosyl phosphatase activity[17]. The B subunits determine the substrate specificity and the cellular localization of the enzyme[18]. Most PP2A holoenzymes are in the cytoplasm, but some B subunits target PP2A to the nucleus[19]. We speculated that HCV proteins might upregulate a nuclear PP2A holoenzyme with phosphotyrosyl phosphatase activity. When we tested liver cell extracts from hepatitis C transgenic mice (B6HCV) for the expression of the catalytic subunit of PP2A, we observed consistently about 30% higher protein expression compared to C57BL/6 control liver extracts (Figure 1B). Not only the expression level of PP2Ac was increased, but also the phosphatase activity, as

determined by an in vitro phosphatase assay (Figure 1C). Since PP2Ac expression and activity are tightly regulated[20-22], the observed differences between HCV transgenic and control mice are likely to be biologically significant.

An influence of the protein phosphatase PP2A on serine or tyrosine phosphorylation of STATs would be a possible mechanism of how PP2A might interfere with Jak-STAT signaling. It was reported that pharmacological inhibition of PP2A induced serine/threonine phosphorylation of STAT3 in human T cells and T lymphoma cell lines and resulted in a functional inhibition of STAT3[23]. Conversely, in 32Dcl3 myeloid progenitor cells, inhibition of PP2A increased tyrosine phosphorylation and nuclear translocation of STAT5 and resulted in a increased STAT5 signaling[24]. In HCV transgenic mice, the overexpression of PP2Ac in liver cells has no effect on STAT1 phosphorylation. Both the tyrosine and serine phosphorylation status of STAT1 from liver extracts of HCV transgenic mice was the same as in control mice (Figure 1D).
Finally, PIAS1 is an obvious candidate protein for interference with STAT1 DNA binding[25]. We therefore analyzed the expression level of PIAS1 in liver extracts of transgenic B6HCV mice and control mice, but found no difference (data not shown).

Example 2: Expression of a constitutively active PP2Ac in human hepatoma cells inhibits Jak-STAT signaling

To further investigate the significance of PP2Ac overexpression in STAT1 signaling we stably transfected human hepatoma derived Huh7 cells with a constitutive active form of PP2Ac, HA-PP2Ac. HA-PP2Ac has been generated by the fusion of an influenza hemagglutinin derived nine-amino-acid epitope tag to the amino terminus of the full length PP2Ac[15]. Transfection of Huh7 cells with HA-PP2Ac resulted in stable expression of the fusion protein without changing the expression level of the endogenous PP2Ac. Although the amount of HA-PP2Ac was less than 10% of the endogenous PP2Ac (Figure 2A), the phosphatase activity was increased more than twofold (Figure 2B). IFNα induced DNA-binding of STAT1 was inhibited in these cells (Figure 2C). Interestingly, the inhibition could be partially reversed by addition of the PP2Ac inhibitor okadaic acid (Figure 2C). Of note, okadaic acid had no effect on STAT1 signaling in untransfected cells (Figure 2C). Again, we found no difference in the tyrosine or in the serine phosphorylation status of STAT1 after IFNα treatment in HA-PP2Ac transfected versus untransfected Huh7 cells (Figure 2D). These findings suggested an active role of PP2A in STAT dephosphorylation in the nucleus. Indeed, it has been reported that the addition of purified PP2Ac to nuclear extracts from IFNγ treated U937 cells inhibits DNA binding of the STAT1 dimer to the GAS oligonucleotide[26]. We therefore tested if the addition of purified, active PP2Ac to nuclear extracts from IFNα treated Huh7 cells could inhibit STAT1 DNA binding in a cell-free system, but found no direct effect of PP2Ac on activated STAT1 in an EMSA with the m67 oligonucleotide (Figure 2E). Therefore, we conclude that dephosphorylation of STAT1 by PP2A is not the mechanism responsible for inhibition of Jak-STAT signaling.

Example 3: Expression of PP2Ac results in arginine hypomethylation of STAT1 and increased association of STAT1 with PIAS1

The association of STAT1 with the inhibitor PIAS1 is regulated by arginine methylation of STAT1 on arginine 31[11]. We therefore determined the arginine methylation status of STAT1 in HA-PP2Ac transfected Huh7 cells by the method described by Mowen

et al. (immunoprecipitation of arginine methylated proteins followed by a Western blot with STAT1 antibodies). Indeed, expression of HA-PP2Ac resulted in a decrease of STAT1 methylation (Figure 3A). Importantly, STAT1 was also hypomethylated in liver extracts of HCV transgenic mice when compared to control mice (Figure 3B and 3C). Methylation of arginine was shown to inhibit the association of STAT1 with PIAS1[11]. Our observation of a decreased level of STAT1 methylation suggests that an enhanced association of PIAS1 with STAT1 might be responsible for the inhibition IFNα induced Jak-STAT signaling. Indeed, the PIAS1-STAT1 association was increased in HA-PP2Ac transfected Huh7 cells (Figure 3D). The effect was almost as pronounced as when PRMT1 was pharmacologically inhibited by 5'-methyl-thioadenosine (MTA) (Figure 3D). We used here MTA treatment as a positive control, since it has been shown that pretreatment with MTA significantly reduced the amount of STAT1 that is immunoreactive with DMA antibodies[11]. Finally, enhanced binding of PIAS1 to STAT1 was also found in liver cell extracts from HCV transgenic mice when compared to controls (Figure 3E). These experiments demonstrate that PP2A is a negative regulator of arginine methylation of STAT1. As a consequence, hypomethylated STAT1 associates with PIAS1, a negative regulator of STAT1 induced gene transcription. This negative regulation is partially dependent on the phosphatase activity of PP2Ac, since inhibition of the catalytic activity of PP2Ac with okadaic acid increased STAT1 methylation (Figure 3F).

Example 4: IFNα induced signaling through STAT1 is inhibited in liver biopsies of patients with chronic hepatitis C

To test if our results obtained in cell lines and transgenic mice are relevant to human disease, we developed a semiquantitative method to measure IFNα induced Jak-STAT signaling in liver biopsies of patients with chronic hepatitis C. Since patients could not be treated with IFNα before liver biopsies were performed, we incubated biopsy samples *ex vivo* with IFNα. We first tested if IFNα was able to readily diffuse in such tissue cylinders and if diffusion was dependent on the degree of liver tissue fibrosis. 5-10 mm long tissue cylinders obtained by percutaneous liver biopsy with a 1.4mm biopsy needle were incubated immediately after retrieval in phosphate buffered saline (PBS) alone or in PBS with 1000U/ml human IFNα. Nuclear translocation of phosphorylated STAT1 was detected throughout the entire biopsy after 10 min, reached a maximum after 20 min, and disappeared after one hour (Figures 4A and 4B). Diffusion of IFNα into the biopsy cylinder was independent of the fibrosis stage of the liver tissue, since the same pattern of uniform STAT1 nuclear translocation was observed in biopsies of patients with liver tissue fibrosis ranging from minimal fibrosis to complete cirrhosis.

The next 44 consecutive biopsies of patients with chronic hepatitis C were used for semiquantitative assessment of IFNα induced STAT1 activation using gel shift assays. Again, biopsy cylinders were incubated in vitro with or without IFNα in PBS for 20 min. Cytoplasmic and nuclear extracts were prepared as detailed under experimental procedures. The samples of 19 of the 44 patients could not be used, because the biopsies were to small and the protein concentration in the nuclear extracts was less than 0.5 mg/ml. From the remaining 25 patients, untreated and IFNα treated samples were analyzed with EMSA (Figure 4C). Signal intensity of the STAT1 gel shift was quantified for each sample, and divided by the protein concentration of the nuclear extracts (normalized gel shift intensity). We then calculated the fold induction of STAT1 gel shifts for each patient by dividing the normalized gel shift intensity values of the IFNα treated biopsy by the normalized value of

the untreated biopsy (Figure 4C). Control samples were obtained from 12 consecutive patients who underwent ultrasound-guided biopsy of focal lesions in otherwise healthy livers (mainly liver metastasis of carcinomas). From the 12 samples, two could not be used because of an insufficient protein concentration in the nuclear extract. Some samples showed activated STAT1 already before stimulation with IFNα (for example patient number 111 in Figure 4C). It is possible that in these patients the Jak-STAT pathway was already activated by endogenous IFN. Alternatively, other STAT1 activating cytokines might have been present in the liver at the time of the biopsy. Some patients with chronic hepatitis C showed no increase in STAT1 DNA binding after treatment (for example patient number 115 in Figure 4C), whereas others had a good response (patient number 73 in Figure 4C). The results are summarized in table 1. Despite this heterogeneity of our findings, as a group, patients with chronic hepatitis C had a poorer response to IFNα treatment than the controls (Figure 4D). Statistical analysis with the Mann-Whitney U test showed a significant difference between controls and HCV samples at $p = 0.03$. We conclude that HCV infection inhibits IFNα induced Jak-STAT signaling in liver cells.

Example 5: Overexpression of PP2A and hypomethylation of STAT1 in chronic hepatitis C

Finally, we extended the analysis of signaling through the Jak-STAT in liver biopsy samples. As in our cell line and transgenic mouse models, we found no inhibition of STAT1 phosphorylation in HCV infected liver cells (Figure 5A). Interestingly, the STAT1 expression level was higher in samples from HCV patients as compared to controls (Figure 5B and 5C). Analyzed with the Mann-Whitney U test, the difference was significant at $p < 0.0001$. PP2Ac was also found to be induced in liver cells of HCV patients (Figure 5D and 5E). The difference was significant at $p = 0.043$ (Mann-Whitney U test). We then measured the PP2A phosphatase activity and found a strong correlation with PP2Ac protein expression levels (data not shown). We conclude that HCV infection induces an upregulation of the catalytically active PP2Ac.

Next, the methylation of STAT1 was tested in large biopsy specimens obtained from two HCV infected patients and two controls. As mentioned, STAT1 expression levels are increased in HCV samples. Nevertheless, less arginine methylated STAT1 could be immunoprecipitated from HCV samples (Figure 5F and 5G). These large amounts of hypomethylated STAT1 avidly bind to PIAS1, as shown in a coimmunoprecipitation of the two proteins (Figure 5H). Thus, despite normal activation of STAT1 by the Jak kinases at the IFN receptors, IFNα induced signaling is severely impaired in HCV infected liver cells, because PIAS1 prevents STAT1 from binding to the promoters of IFN target genes.

To complete our analysis, the expression of the STAT1 phosphatase TC-PTP was determined. No difference was detected between extracts from biopsies of HCV infected patients and controls (Figure 5I).

Example 6: Treatment with a methyl group donor restores STAT1 methylation and IFN signaling.

Huh7 cells and Huh7 cells stably transfected with HA-PP2Ac were treated with various combinations of hIFNa and AdoMet (SAMe). In the HA-PP2Ac cells IFN signaling and STAT1 methylation were impaired in comparison with the untransfected cells. Moreover, the HA-PP2Ac cells showed increased association between STAT1 and Pias1, indicating inhibition of STAT1-mediated signaling. Treatment with the methyl

group donor SAMe (or AdoMet) at concentrations as low as 1.7 nM essentially eliminated the effect of HA-PP2A transfection with regard to STAT1 methylation, STAT1 association with Pias1, and signaling via hIFNa. Thus, SAMe, by restoring STAT1 methylation, restored responsiveness to IFN in this model of hepatitis C infection.

While the present invention has been described with reference to certain preferred embodiments, one of ordinary skill in the art will recognize that additions, deletions, substitutions, modifications and improvements can be made while remaining within the spirit and scope of the present invention as defined by the appended claims.

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Table 1

Liver biopsies from patients with chronic hepatitis C and from controls

Patient number	Sex	Age	Diagnosis	HCV Geno-type	Viral Load (IU/ml)	Response to PegIFN-Ribavirin	Liver Biopsy: Grade	Liver Biopsy: Stage	EMSA (fold increase)	PP2Ac (arbitrary units)	STAT1 (arbitrary units)
64	f	35	control				mild	minimal	3.3	482	118
83	m	54	control				none	none	4.7	1752	0
133	f	43	control				none	none	1.3	653	799
89	f	62	control				mild	none	12	2445	295
77	m	36	control				mild	none	3.1	0	0
104	m	36	control				mild	none	2.3	933	0
136	f	59	control				mild	none	8.5	931	393
159	f	67	control				mild	none	2.1	1630	2642
164	f	62	control				mild	none	0.9	1376	2006
170	m	82	control				none	none	3.2	339	4211
160	m	33	HCV	1a	397000	ETR	moderate	moderate	1.3	5018	3950
165	f	39	HCV	1a	664000	ongoing	mild	mild	1.4	2216	7416
156	f	32	HCV	1a	>850000	ETR	moderate	moderate	0.9	4959	5693
139	m	30	HCV	1a	1900000	ETR	moderate	moderate	3	656	5149
75	f	43	HCV	1a	346401	NR	mild	severe	7.3	0	6846
169	m	29	HCV	1b	>500000	ongoing	mild	mild	0.9	5121	8114
114	m	58	HCV	1b	1420000	NR	severe	cirrhosis	1.7	14436	5344
132	f	32	HCV	1b	24400	ETR	moderate	moderate	3.5	8765	7198
102	m	57	HCV	1b	3090000	NR	moderate	mild	2.5	2031	8377
73	f	62	HCV	2a	12800000	SR	severe	cirrhosis	4.1	0	795
111	m	45	HCV	2a		not treated	moderate	moderate	1	567	5976
109	m	43	HCV	2b	1450000	NR	moderate	severe	2	1087	1983
55	f	69	HCV	3		relapse	moderate	cirrhosis	0.5	1587	10556
71	m	22	HCV	3a	608000	not treated	moderate	moderate	1.2	0	1765
97	f	49	HCV	3a	240000	NR	moderate	severe	2.2	10807	6873
103	m	36	HCV	3a	282000	relapse	moderate	mild	1.3	4463	4255
149	m	38	HCV	3a	330000	SR	mild	mild	0.8	571	14459
119	f	46	HCV	3a	489000	ETR	mild	mild	12	8762	2822
69	m	36	HCV	3a	545000	not treated	moderate	moderate	0.9	1027	3964
138	f	34	HCV	3a	55300	SR	moderate	none	0.8	1199	7631
115	m	49	HCV	4c	>850000	ongoing	severe	moderate	2.5	12246	8235
92	m	49	HCV	4c	251000	ongoing	severe	mild	0.5	6145	11787

107	m	44	HCV	4c	370000	relapse	mild	cirrhosis	1.5	12085	8144
144	f	48	HCV	4c	684000	not treated	mild	mild	1	5667	8477
134	m	46	HCV	2a	880000	ongoing	mild	severe	2.6	1312	4428

What is claimed is:

1. A compound increasing methylation of a STAT1 in vivo.

2. A compound according to claim 1, wherein said compound acts by increasing or decreasing an activity of a protein selected from the group consisting of: PP2A2, PRMT1, and PIAS1.

3. A therapeutic composition comprising said compound of claim 2 and a pharmacologically-acceptable carrier.

4. A compound decreasing activity of a PP2A.

5. A therapeutic composition comprising said compound of claim 4 and a pharmacologically-acceptable carrier.

6. A method of selecting a drug to treat hepatitis C, comprising the steps of:

a. providing a plurality of drug candidates,

b. assaying the candidates to ascertain a change in a level of STAT1 methylation upon application of the candidates to a model system,

c. selecting candidates that increase methylation of STAT1, and

d. selecting a candidate from among those selected in step c that is effective in treating hepatitis C.

7. The method of claim 6, wherein said model system is selected from the group consisting of a protein binding assay, a protein activity assay, a cell culture model, and an animal model.

8. The method of claim 6, wherein step d comprises testing said drug candidate in an animal model of hepatitis C infection.

9. A drug developed by the method of claim 8.

10. A method of selecting a drug to treat hepatitis C, comprising the steps of:

a. providing a plurality of drug candidates,

b. assaying the candidates to ascertain a change in a level of PP2A activity upon application of the candidates to a model system,

c. selecting candidates that change the level of PP2A activity, and

d. selecting a candidate from among those selected in step c that are effective in treating hepatitis C.

11. The method of claim 10, wherein said model system is selected from the group consisting of a protein binding assay, a protein activity assay, a cell culture model, and an animal model.

12. The method of claim 10, wherein step d comprises testing said drug candidate in an animal model of hepatitis C infection.

13. A drug selected by the method of claim 12.

14. A method of treating hepatitis C infection, comprising enhancing a level of methylation of STAT1.

15. The method of claim 14, further comprising increasing or decreasing an activity of a protein selected from the group consisting of: PPA2, PRMT1, and PIAS1.

16. The method of claim 15, wherein said increasing or decreasing step is carried out by administering a therapeutic composition.

17. A method of treating hepatitis C infection, comprising reducing an activity of PP2A.

18. The method of claim 17, wherein said reducing is carried out by administering a therapeutic composition.

19. A method according to claim 14, further comprising the step of administering a drug selected by the method of claim 6.

20. A method according to claim 14, further comprising the step of administering a drug selected by the method of claim 10.

21. The use of methyl group donor for the manufacture of a pharmaceutical composition, wherein the pharmaceutical composition comprises a methyl-group donor in an amount effective to increase a level of STAT1 methylation in a patient.

22. The use of a methyl group donor to increase a level of STAT1 methylation in a patient, comprising (1) selecting a patient, and (2) treating the patient with a methyl group donor in an amount effective to increase the level of STAT1 methylation in the patient.

23. The method of claim 22, further comprising the step of monitoring said level of STAT1 methylation in said patient.

24. A pharmaceutical composition consisting essentially of interferon, ribavarin, a methyl group donor, and a pharmacologically-acceptable carrier.

25. Any of claims 21-24 wherein said methyl group donor is SAMe.

26. Any of claims 21-25 wherein betaine is used in conjunction with said methyl group donor.

27. A method of monitoring a patient, comprising the steps of (1) selecting a patient undergoing treatment for viral infection, and (2) testing one or more samples from the patient to ascertain a level of STAT1 methylation.

28. The method of claim 27, wherein said viral infection is a hepatitis C infection.

29. A pharmaceutical composition comprising pegylated interferon, ribavarin, SAMe, betaine, and a pharmacologically-acceptable carrier.

Fig 1

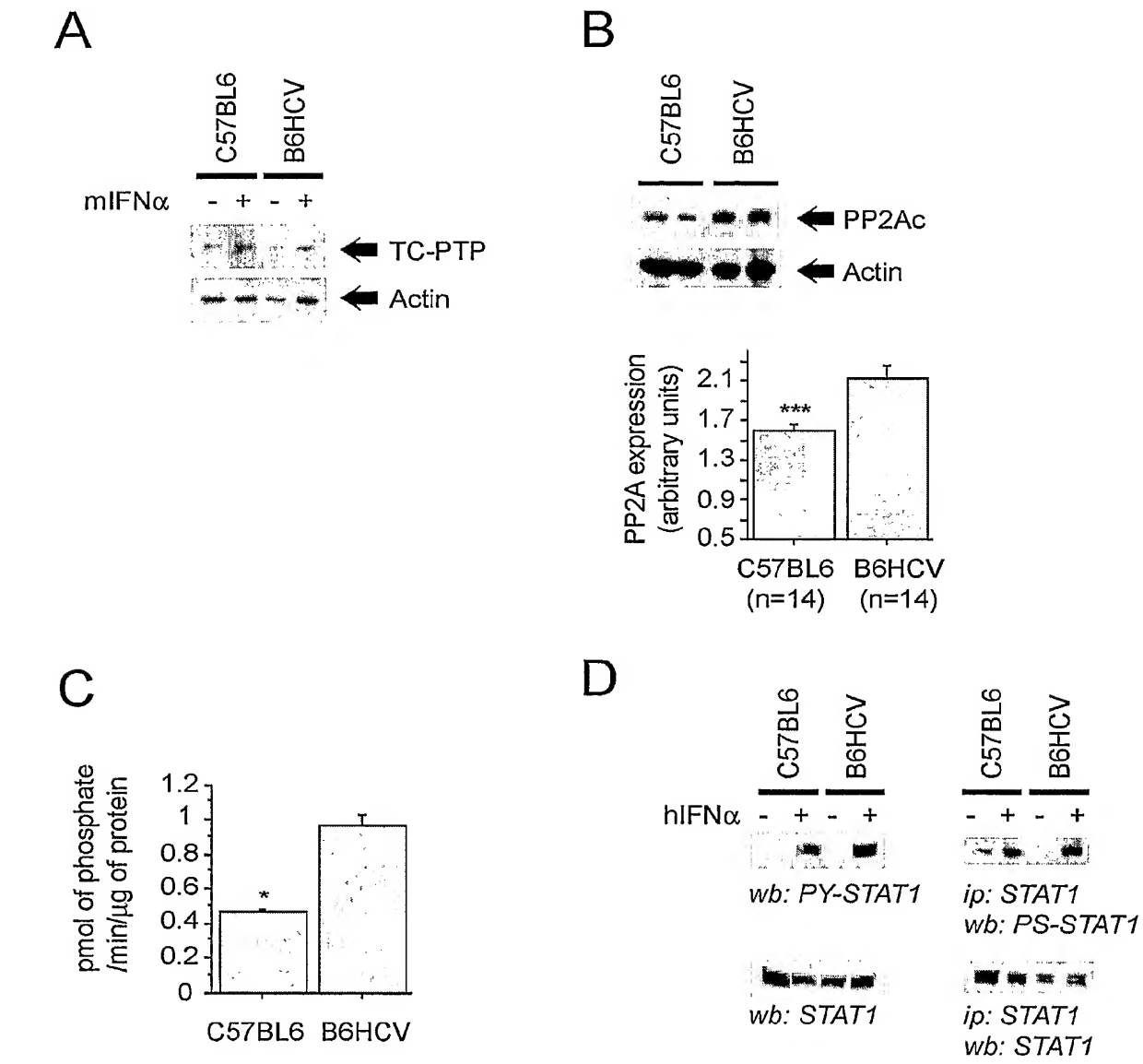

A
C57BL6
B6HCV
mIFNα - + - +
TC-PTP
Actin
B
C57BL6
B6HCV
PP2Ac
Actin
PP2A expression (arbitrary units)
2.1
1.7
1.3
0.9
0.5
***
C57BL6 (n=14)
B6HCV (n=14)
C
pmol of phosphate /min/µg of protein
1.2
1
0.8
0.6
0.4
0.2
0
*
C57BL6
B6HCV
D
C57BL6
B6HCV
hIFNα - + - +
wb: PY-STAT1
wb: STAT1
ip: STAT1
wb: PS-STAT1
ip: STAT1
wb: STAT1

Fig 2

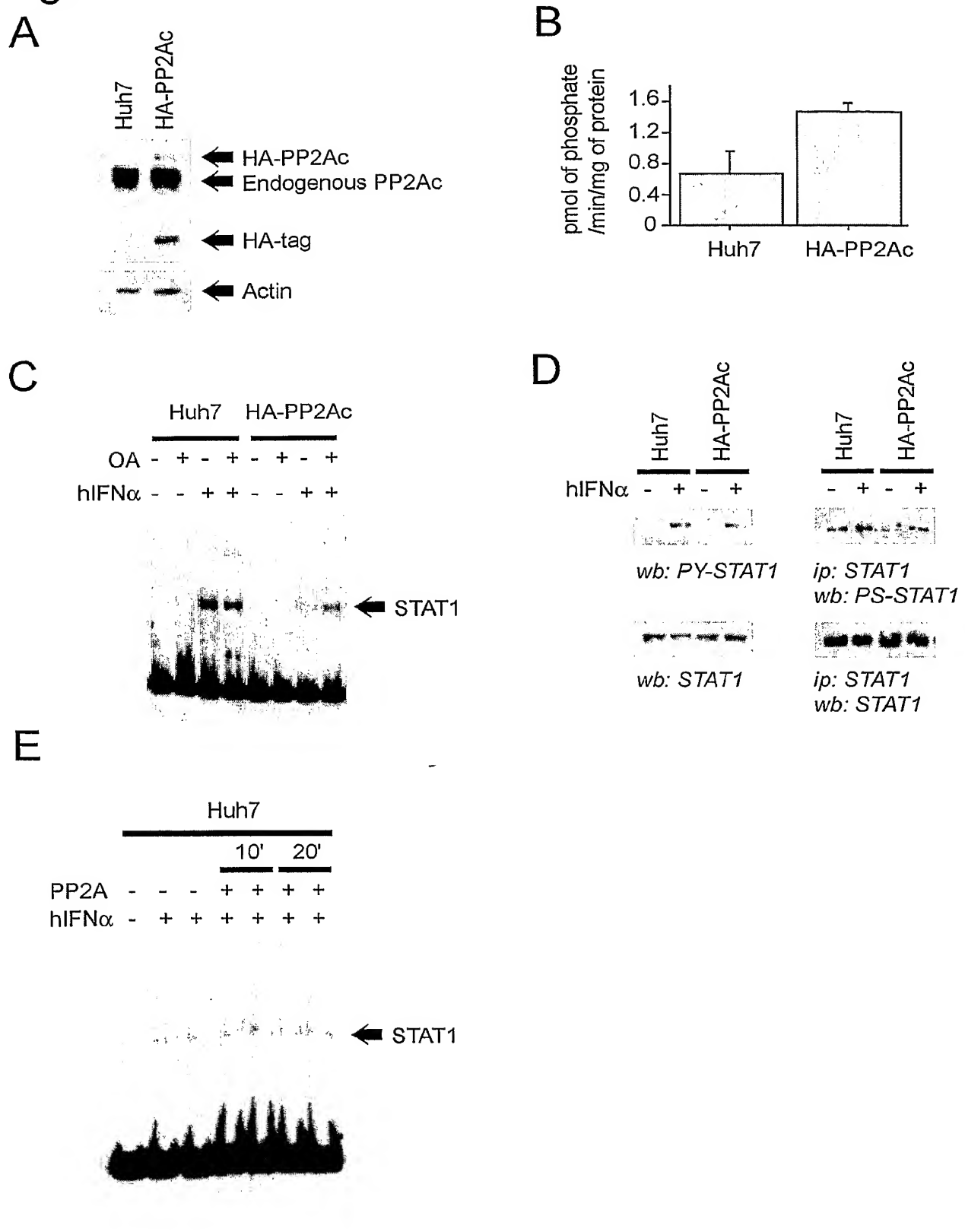

A
Huh7
HA-PP2Ac
HA-PP2Ac
Endogenous PP2Ac
HA-tag
Actin
B
pmol of phosphate /min/mg of protein
1.6
1.2
0.8
0.4
0
Huh7
HA-PP2Ac
C
Huh7
HA-PP2Ac
OA - + - + - + - +
hIFNα - - + + - - + +
STAT1
D
Huh7
HA-PP2Ac
hIFNα - + - +
wb: PY-STAT1
wb: STAT1
ip: STAT1
wb: PS-STAT1
ip: STAT1
wb: STAT1
E
Huh7
10'
20'
PP2A - - - + + + +
hIFNα - + + + + + +
STAT1

Fig 3

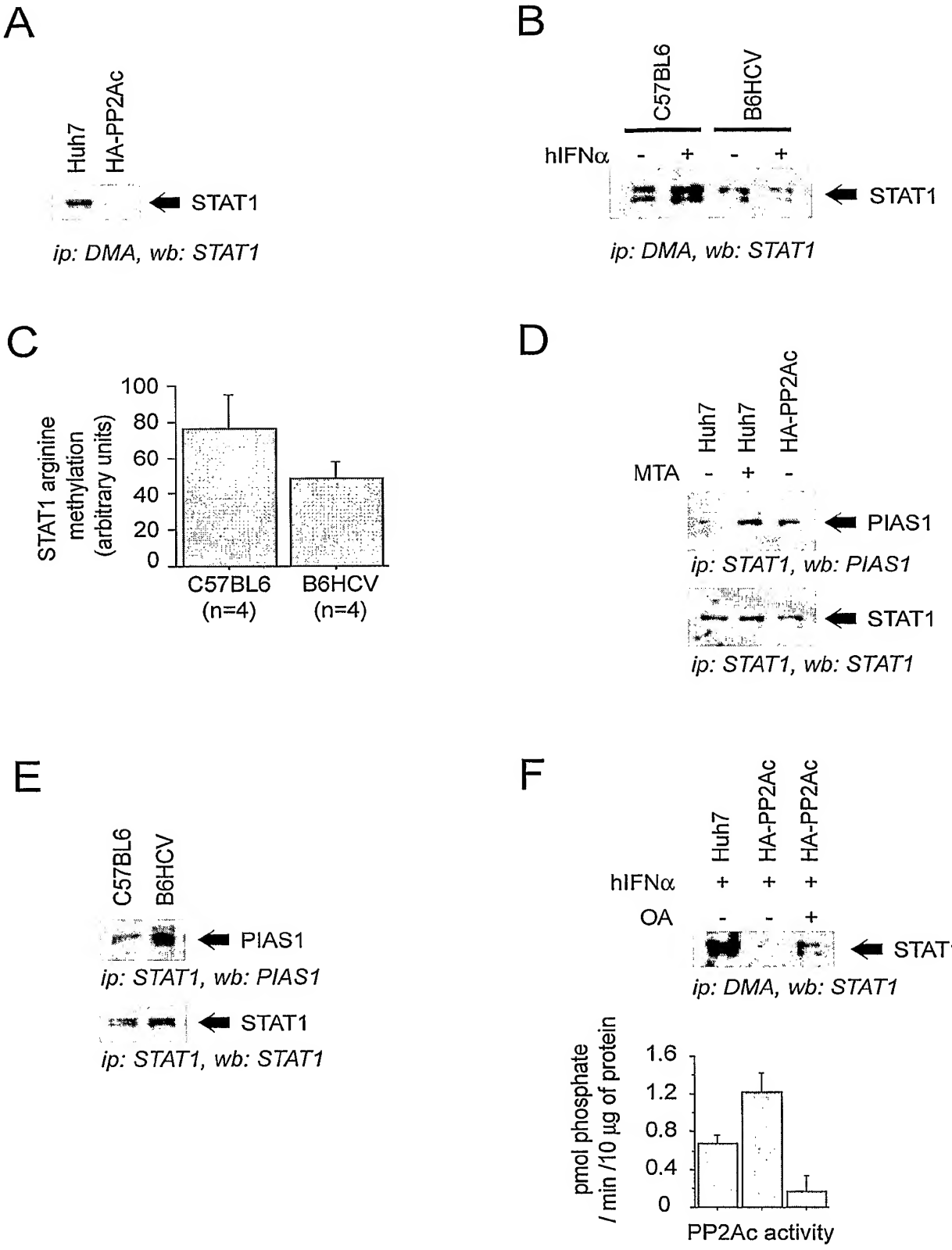

A
Huh7
HA-PP2Ac
STAT1
ip: DMA, wb: STAT1
B
C57BL6
B6HCV
hIFNα - + - +
STAT1
ip: DMA, wb: STAT1
C
STAT1 arginine methylation (arbitrary units)
100
80
60
40
20
0
C57BL6 (n=4)
B6HCV (n=4)
D
Huh7
Huh7
HA-PP2Ac
MTA - + -
PIAS1
ip: STAT1, wb: PIAS1
STAT1
ip: STAT1, wb: STAT1
E
C57BL6
B6HCV
PIAS1
ip: STAT1, wb: PIAS1
STAT1
ip: STAT1, wb: STAT1
F
Huh7
HA-PP2Ac
HA-PP2Ac
hIFNα + + +
OA - - +
STAT1
ip: DMA, wb: STAT1
pmol phosphate / min /10 µg of protein
1.6
1.2
0.8
0.4
0
PP2Ac activity

Fig 4

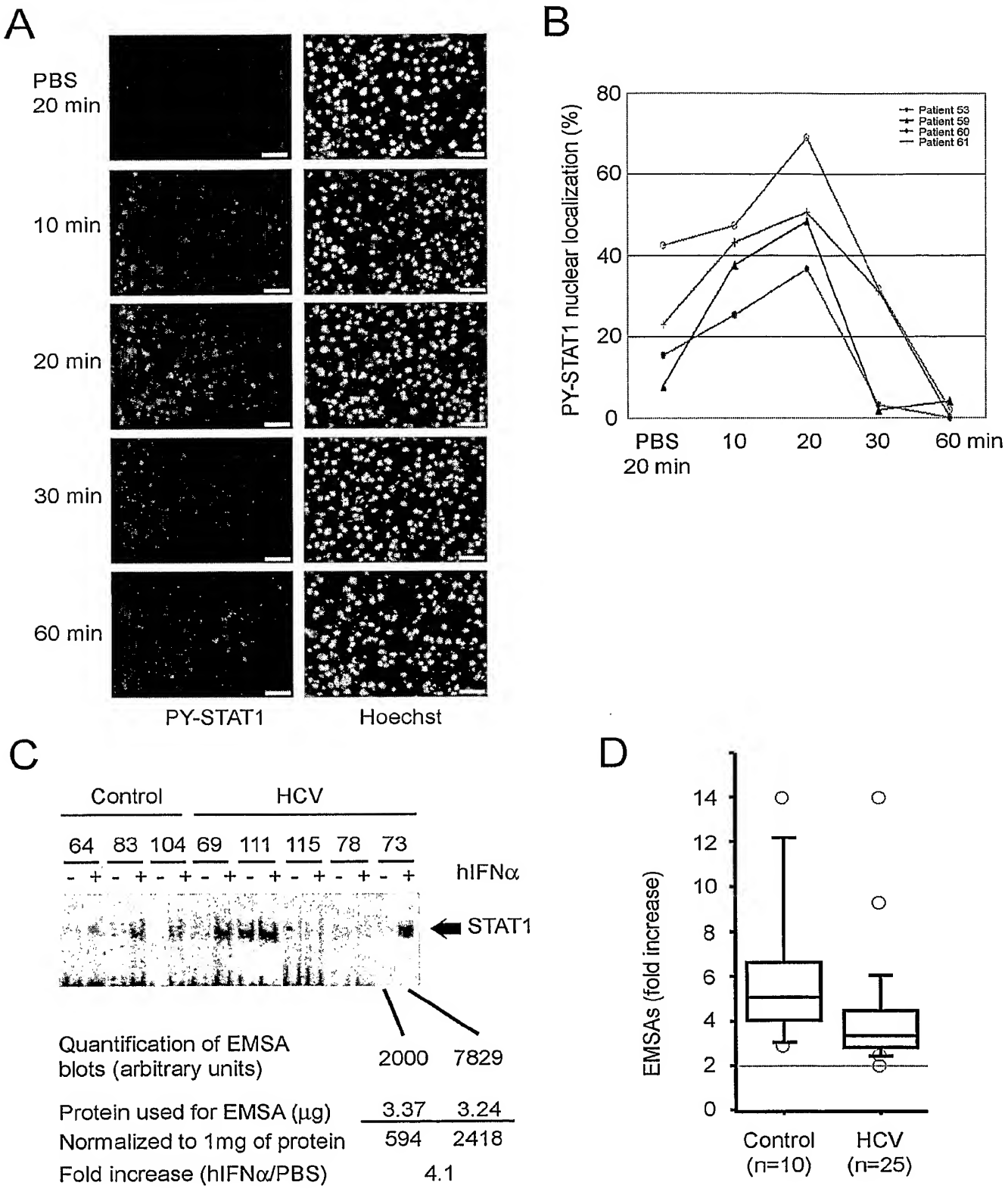

A
PBS
20 min
10 min
20 min
30 min
60 min
PY-STAT1
Hoechst
B
PY-STAT1 nuclear localization (%)
80
60
40
20
0
Patient 53
Patient 59
Patient 60
Patient 61
PBS
20 min
10
20
30
60 min
C
Control
HCV
64 83 104 69 111 115 78 73
- + - + - + - + - + - + - + - +
hIFNα
STAT1
Quantification of EMSA blots (arbitrary units)
2000 7829
Protein used for EMSA (µg) 3.37 3.24
Normalized to 1mg of protein 594 2418
Fold increase (hIFNα/PBS) 4.1
D
EMSAs (fold increase)
14
12
10
8
6
4
2
0
Control (n=10)
HCV (n=25)

Fig 5

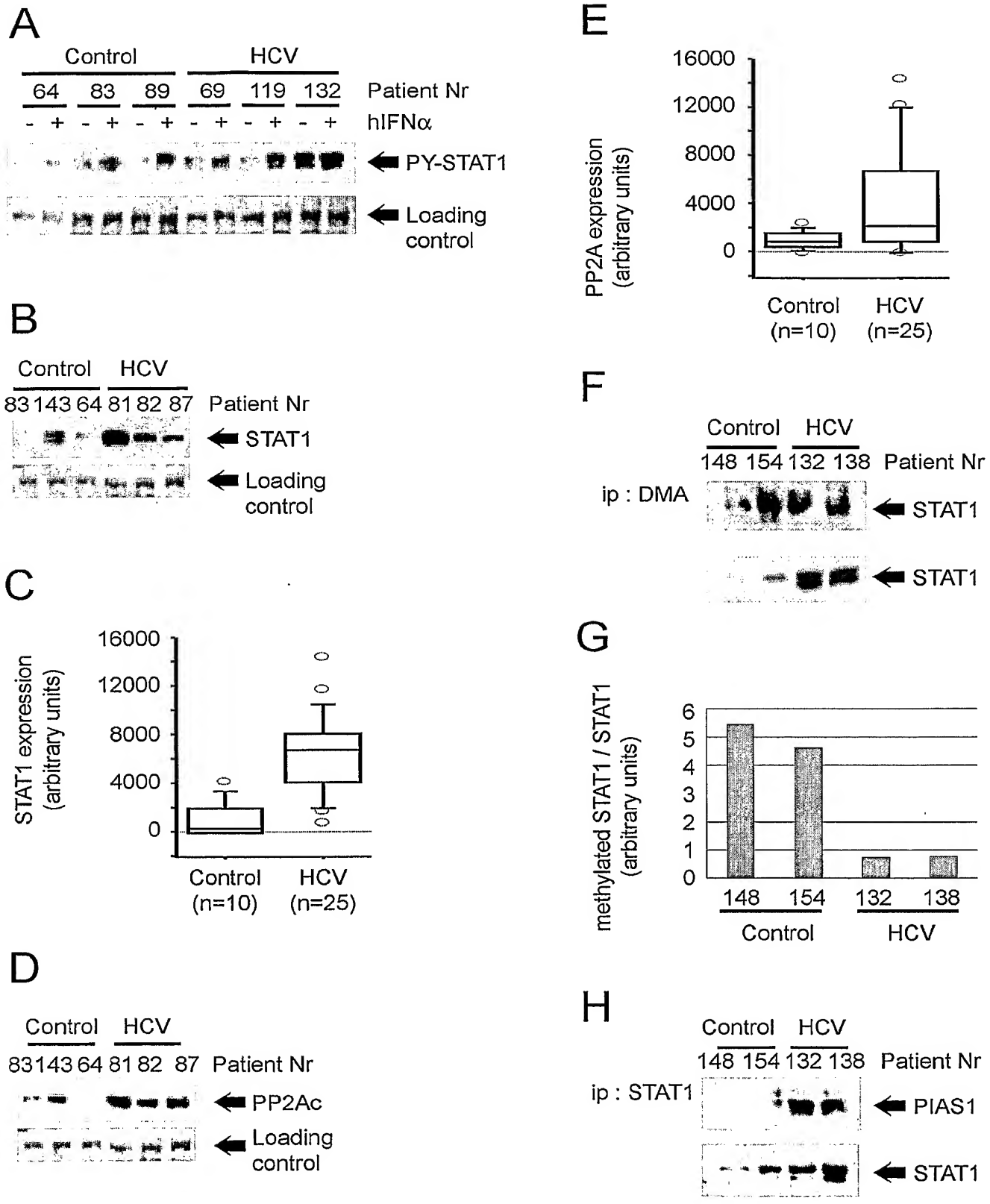

A
Control
HCV
64 83 89 69 119 132
Patient Nr
- + - + - + - + - + - +
hIFNα
PY-STAT1
Loading control
B
Control HCV
83 143 64 81 82 87
Patient Nr
STAT1
Loading control
C
STAT1 expression (arbitrary units)
16000
12000
8000
4000
0
Control (n=10)
HCV (n=25)
D
Control HCV
83 143 64 81 82 87
Patient Nr
PP2Ac
Loading control
E
PP2A expression (arbitrary units)
16000
12000
8000
4000
0
Control (n=10)
HCV (n=25)
F
Control HCV
148 154 132 138
Patient Nr
ip : DMA
STAT1
STAT1
G
methylated STAT1 / STAT1 (arbitrary units)
6
5
4
3
2
1
0
148 154 132 138
Control
HCV
H
Control HCV
148 154 132 138
Patient Nr
ip : STAT1
PIAS1
STAT1

Fig 5 cont

I

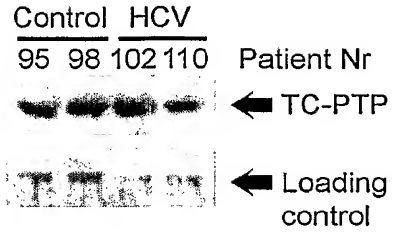


Fig 6

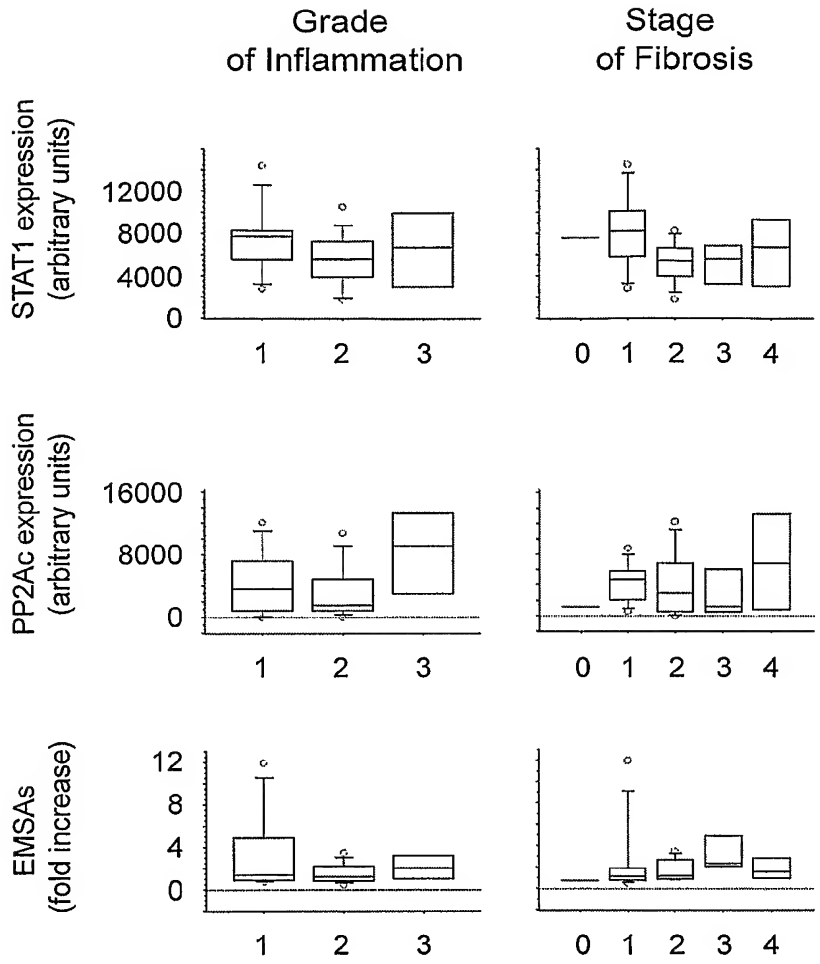

Grade of Inflammation
Stage of Fibrosis
STAT1 expression (arbitrary units)
12000
8000
4000
0
1 2 3
0 1 2 3 4
PP2Ac expression (arbitrary units)
16000
8000
0
1 2 3
0 1 2 3 4
EMSAs (fold increase)
12
8
4
0
1 2 3
0 1 2 3 4

Fig 7

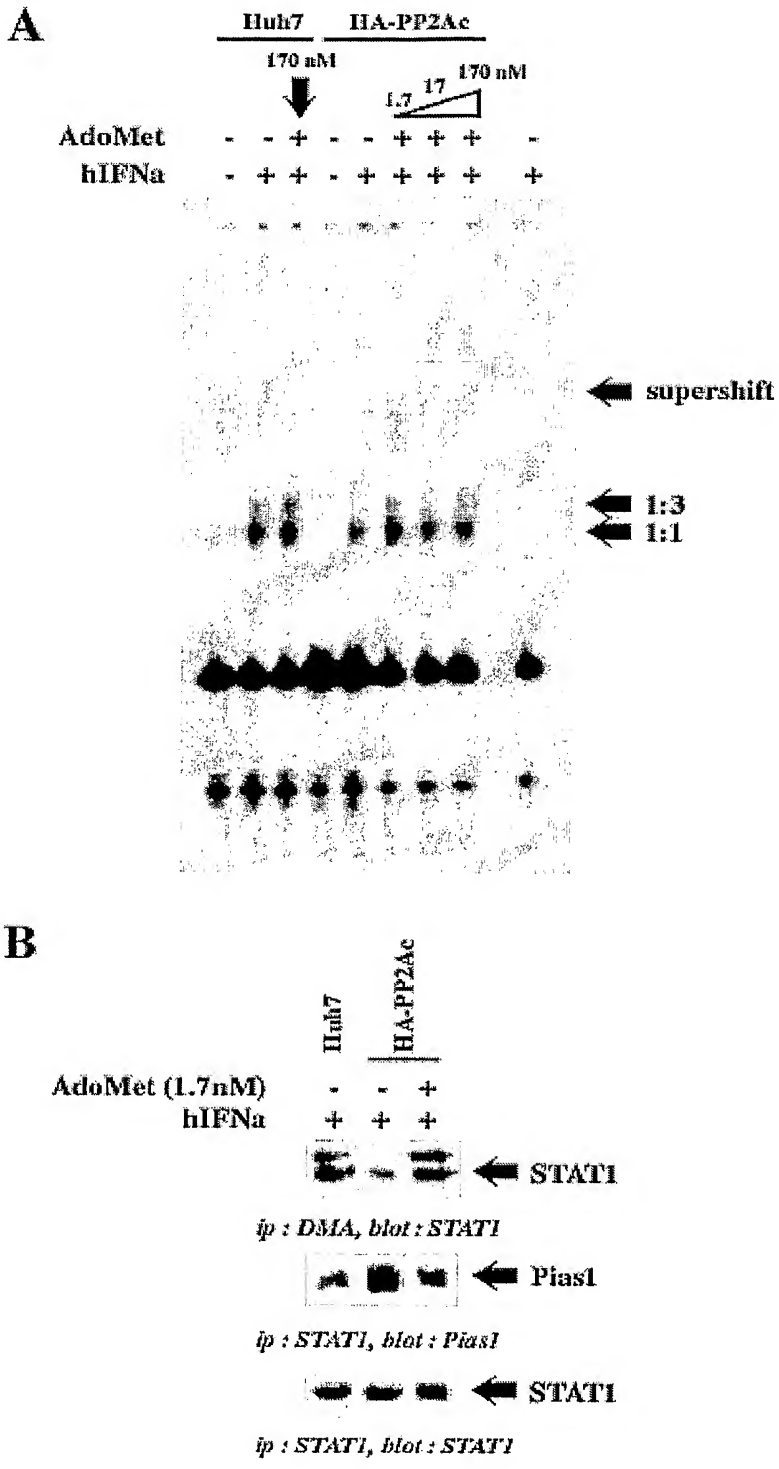
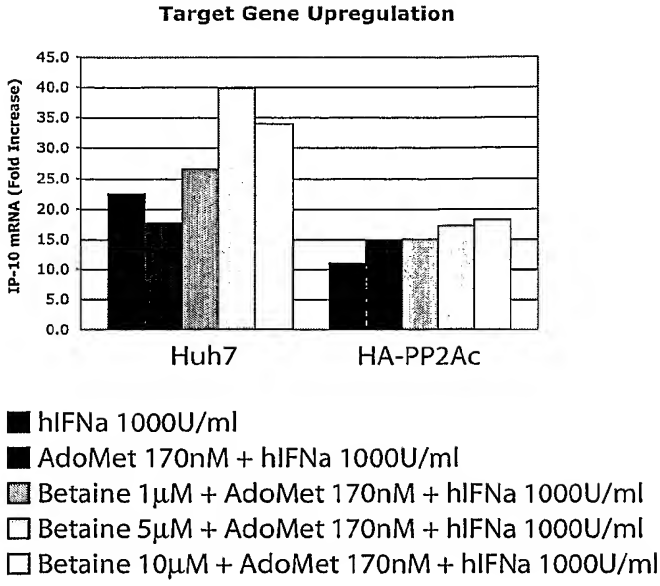

A
Huh7
HA-PP2Ac
170 nM
1.7
17
170 nM
AdoMet
hIFNa
supershift
1:3
1:1
B
Huh7
HA-PP2Ac
AdoMet (1.7nM)
hIFNa
STAT1
ip : DMA, blot : STAT1
Pias1
ip : STAT1, blot : Pias1
STAT1
ip : STAT1, blot : STAT1

Figure 8


Target Gene Upregulation
IP-10 mRNA (Fold Increase)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Huh7
HA-PP2Ac
hIFNa 1000U/ml
AdoMet 170nM + hIFNa 1000U/ml
Betaine 1µM + AdoMet 170nM + hIFNa 1000U/ml
Betaine 5µM + AdoMet 170nM + hIFNa 1000U/ml
Betaine 10µM + AdoMet 170nM + hIFNa 1000U/ml

Interr ıl Application No
PCT/IB2005/000158

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/42 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/83754 A (IMMUSOL, INCORPORATED) 8 November 2001 (2001-11-08) claims 21-31	6-12
Y	DUONG FRANCOIS H T ET AL: "Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A." GASTROENTEROLOGY, vol. 126, no. 1, 1 January 2004 (2004-01-01), pages 263-277, XP002331956	6-12
Y	page 275, right-hand column, line 18 - page 276, left-hand column, line 3	27,28
Y	WO 01/41782 A (CHIRON CORPORATION) 14 June 2001 (2001-06-14) example 2	27,28

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2005	27/06/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gunster, M

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/024461 A (SCHERING CORPORATION) 27 March 2003 (2003-03-27) cited in the application	21,24,25
Y	claims 2,4	26,29
X	WO 03/030929 A (TRANSITION THERAPEUTICS INC) 17 April 2003 (2003-04-17)	21
Y	page 13, line 15 - line 28; claim 65	26,29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2005/000158

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14-20, 22, 23
because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

2. [X] Claims Nos.: 1-5,13
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Claims Nos.: 14-20, 22, 23

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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Continuation of Box II.2

Claims Nos.: 1-5,13

Present claims 1-5 and 13 relate to a compound defined by reference to a desirable characteristic or property, namely. being able to increase STAT1 methylation or decrease PP2A activity or being identifyable by teh method of claim 12.

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for none of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for claims 1-5 and 13.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/IB2005/000158

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0183754 A	08-11-2001	US 6808876 B1	26-10-2004
		AU 5944101 A	12-11-2001
		CA 2409219 A1	08-11-2001
		EP 1278845 A2	29-01-2003
		WO 0183754 A2	08-11-2001
		US 2005064396 A1	24-03-2005
WO 0141782 A	14-06-2001	AU 2069801 A	18-06-2001
		CA 2393688 A1	14-06-2001
		EP 1237567 A2	11-09-2002
		JP 2003516360 T	13-05-2003
		NO 20022731 A	08-08-2002
		WO 0141782 A2	14-06-2001
		US 2002141971 A1	03-10-2002
		US 2001043915 A1	22-11-2001
WO 03024461 A	27-03-2003	WO 03024461 A1	27-03-2003
		US 2003055013 A1	20-03-2003
WO 03030929 A	17-04-2003	US 2003152552 A1	14-08-2003
		US 2003086901 A1	08-05-2003
		WO 03030929 A1	17-04-2003